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Assessment of the Allosteric Interactions of the Bisquaternary Heptane-1,7-bis(dimethyl-3′-phthalimidopropyl)ammonium Bromide at M_1 and M_2 Muscarine Receptors

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SUMMARY

The interaction of the allosteric muscarine receptor antagonist heptane-1,7-bis(dimethyl-3′-phthalimidopropyl)ammonium bromide (C_7/3-phth) with M_1 muscarine receptors in rat cerebral cortex and rabbit vas deferens and M_2 muscarine receptors in guinea pig atria was investigated. In atria, C_7/3-phth completely inhibited the dissociation of *N*-[^{3}H]methylscopolamine ([^{3}H]NMS) in the presence of excess unlabeled NMS and slowed the washout of NMS in functional experiments. C_7/3-phth also produced supra-additive inhibition of the negative inotropic effects of carbachol when combined with NMS. This latter phenomenon was less pronounced when pirenzepine (PZP) was used in place of NMS. Cooperativity factors for the interaction of C_7/3-phth with other antagonists were obtained by fitting the data to a theoretical model for interaction between an agonist, a competitive antagonist, and an allosteric antagonist. The values obtained indicate that C_7/3-phth exhibits a greater degree of negative heterotropic cooperativity with PZP than with NMS at the M_2 muscarine receptor. In the rat cerebral cortex, C_7/3-phth slowed the dissociation of [^{3}H]NMS and [^{3}H]quinuclidinyl benzilate from the M_1 receptor to the same extent but appeared not to affect the dissociation of [^{3}H]PZP. In rabbit vas deferens, the inhibitory effect of the combination of C_7/3-phth and atropine on the responses to McN-A-343 at the M_1 receptor was more pronounced than that of the combination of C_7/3-phth and PZP. Comparison of the findings for both central and peripheral M_1 receptors with those obtained for the cardiac M_2 receptor suggests that the allosteric interaction of C_7/3-phth is less evident at the M_1 receptor, particularly in the case of PZP.

The bisquaternary compound C_7/3-phth is one of a series of hexamethonium derivatives that have been shown to possess potent muscarine receptor-blocking properties (1, 2). Functional studies have demonstrated that this compound possesses high affinity for the M_2 muscarine receptor present in cardiac tissue. It showed a 32-fold lower affinity for the M_3 muscarine receptor present in guinea pig ileum (3) and a 3-fold lower affinity for the prejunctional M_1 muscarine receptor in rabbit vas deferens (4). In rat cortex, C_7/3-phth was found to possess a 4.5-fold lower affinity for M_1 receptors than for M_2 receptors, whereas the K_i value observed for this compound at putative M_4 receptors in rabbit lung was similar to the value at M_2 muscarine receptors in rat atria (5).

C_7/3-phth has also been shown to act allosterically at muscarine receptors (1–3), a phenomenon observed with other compounds known to exhibit a high affinity for M_2 receptors, such as gallamine (6), AF-DX 116 (7), himbacine (8), and methoctramine (9). In support of an allosteric interaction, Arunlakshana-Schild plots of the effect of C_7/3-phth on cardiac tissue were nonlinear (1) and, in combination with atropine (1) and other competitive muscarine receptor antagonists such as homatropine and dexetimide (2), C_7/3-phth produced a greater antimuscarinic effect than that predicted for the combination of two competitive antagonists.

Allosteric interactions were also observed in guinea pig ileum, where combination experiments with C_7/3-phth and atropine or homatropine produced a supra-additive inhibitory effect against the responses to CCh (3). Radioligand kinetic studies performed in both guinea pig atrial and ileal tissues demonstrated that the dissociation rate of the nonselective muscarine receptor antagonist [^{3}H]QNB was significantly different for the combination of atropine and C_7/3-phth than for atropine alone (3).

The aim of the present study was to further characterize the allosteric properties of C_7/3-phth at various muscarine receptor sites both in the central nervous system and in the periphery, using radioligand binding methods as well as functional organ bath studies.

ABBREVIATIONS: C_7/3-phth, heptane-1,7-bis(dimethyl-3′-phthalimidopropyl)ammonium bromide; NMS, *N*-methylscopolamine; CCh, carbachol; PZP, pirenzepine; QNB, quinuclidinyl benzilate; McN-A-343, (4-*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride; AF-DX 116, 11-[[2-(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepine-6-one; C/R, concentration/response; DR, dose ratio; W84, hexamethylene-1,6-bis(dimethyl-3′-phthalimidopropyl)ammonium bromide.

Materials and Methods

Radioligand dissociation rate kinetic studies. Hooded Wistar rats of either sex were killed by decapitation and their brains were rapidly removed and placed in ice-cold phosphate buffer (50 mM Na_2HPO_4, pH 7.4). After dissection, the cortex was blotted dry, weighed, and homogenized in 15 volumes of the phosphate buffer using a Potter Elvehjem homogenizer (20 strokes).

Homogenates were centrifuged at −4° for 10 min at 1000 × *g*, using a Sorvall RC-2B refrigerated centrifuge. The supernatant (S_1 fraction) was recentrifuged for two 20-min periods at 40,000 × *g*. The pellet from this centrifugation (P_2 fraction) was resuspended in ice-cold phosphate buffer (5 ml/g of original wet weight) and used for the binding studies. Radioligand binding assays were performed using either [^{3}H]QNB, [^{3}H]PZP, or [^{3}H]NMS.

K_d values obtained from Scatchard analysis (10, 11) were as follows: [^{3}H]QNB, 46.1 pM (95% confidence interval, 39.3–54.1; three experiments) (B_{max} = 1214 ± 397 fmol/mg of protein, mean ± standard error); [^{3}H]PZP, 3.22 nM (1.05–9.87; three experiments) (B_{max} = 942 ± 411 fmol/mg of protein); [^{3}H]NMS, atria, 0.27 nM (B_{max} = 167 ± 20 fmol/mg of protein); cortex, 0.11 nM (0.05–0.23, three experiments) (B_{max} = 878 ± 247 fmol/mg of protein)) (5, 12).

For the kinetic studies, 0.2 ml of cortical homogenate was added to incubation tubes and allowed to equilibrate with a fixed concentration of radioligand (50 pM [^{3}H]QNB, 0.3 nM [^{3}H]NMS, or 3 nM [^{3}H]PZP) at 32° for 1 hr. After this period, unlabeled (nonradioactive) antagonist (~30–300 times the K_d value) was added alone or in combination with C_7/3-phth (10 μM), to inhibit the reassociation of the radioligand. After this procedure, the amount of radioactivity was determined at various time intervals to assess the rate of dissociation of the radioligand.

Other kinetic experiments were conducted using guinea pig atria. Hearts were placed in ice-cold physiological saline solution (0.9%, w/v, NaCl) and were flushed to remove blood from the coronary arteries. Atria were then separated from ventricles, blotted dry, minced finely, weighed, mixed with 15 volumes of phosphate buffer, and homogenized in an Ultra-Turrax set at 0.75 times the maximum speed for 2 × 30 sec. After centrifugation at −4° for 10 min at 1000 × *g*, the S_1 fraction was collected and used in subsequent radioligand dissociation rate studies. Previous studies have shown that use of this fraction with cardiac tissue gives similar binding characteristics as does a P_2 fraction (13).

In all cases, incubation was terminated by filtration through Whatman GF/B filters (presoaked in phosphate buffer containing 10 μM atropine and 0.5% polyethylenimine) positioned on a Brandel cell harvester. Filters were washed three times with 4-ml aliquots of ice-cold phosphate buffer and placed in plastic vials with 5 ml of Filter Count (Packard) for radioactivity measurements. Specific binding was defined using atropine (10 μM). In all cases, total binding was <10% of added radioligand.

Organ bath studies. Guinea pig left atria were removed as described above, placed in a 20-ml organ bath in McEwen's solution (14) at 37°, and bubbled with carbogen (95% O_2/5% CO_2). Inotropic responses to electrical stimulation at 3 Hz, 1 msec, and 1.5 times the maximal voltage were measured via a Grass force-displacement transducer (FT.03) connected to a Grass model 79D polygraph. The tissue was allowed to equilibrate for 30 min under a resting tension of 1 g. Sections of rabbit vas deferens were set up for transmural stimulation under 1-g tension at 31° as described by Eltze (15), with modifications as outlined by Choo and Mitchelson (4).

Antagonist combination experiments. C/R curves for the negative inotropic effects of CCh in guinea pig atria were established. A minimum of three concentrations of the agonist, producing between 20% and 80% of the maximum responses, were used to determine each curve, with responses being duplicated over a period of 20–40 min. The bath was washed through twice with fresh McEwen's solution after each dose of agonist and a period of 5 min was allowed between each addition of drug.

The C/R curves were then re-established after a 1-hr incubation of the tissue with different concentrations of NMS, PZP, or C_7/3-phth in duplicate, as described above. After establishment of these second curves (with either NMS or PZP), 10 μM C_7/3-phth was equilibrated with the tissue for an additional 60 min in the presence of a fixed concentration of either NMS or PZP and a third series of C/R curves for CCh were determined.

In the rabbit vas deferens, cumulative C/R curves were established for the inhibitory effect of McN-A-343 on contractile responses to electrical stimulation. Each concentration of agonist was applied for 5 min. A washout and recovery period of 30 min was allowed between generation of each curve.

After establishment of reproducible C/R curves for McN-A-343, an antagonist (either atropine or PZP) was allowed to incubate with the tissue for 45 min before the effect of McN-A-343 was re-evaluated. After the C/R curves in the presence of the first antagonist were established, C_7/3-phth (10 μM) was then added to the organ bath without removal of the first antagonist and a new series of C/R curves for McN-A-343 were determined. In other experiments, the effect of C_7/3-phth (10 μM) alone on the responses to McN-A-343 was determined as described above.

Washout experiments. In some experiments with guinea pig atria, C/R curves for CCh in the presence of NMS were established as described above. Progressive washout of NMS from the tissue was then undertaken. The recovery from NMS inhibition was assessed by using a single concentration of CCh, which approximated a 50% response, to re-establish the position of the C/R curve at various times after commencement of washout (assuming that the C/R curve remained parallel to the original curve). From the leftward shifts monitored over this period, DRs were calculated to assess the "apparent" concentration of NMS remaining in the tissue and the percentage receptor occupancy. In experiments where equilibrium was established with a concentration of NMS in combination with C_7/3-phth (10 μM), C_7/3-phth remained in the solution during the washout of NMS.

Drugs used. Drugs used were (−)-[^{3}H]QNB and [^{3}H]NMS (Amersham, Amersham, UK), [^{3}H]PZP (NEN, Wilmington, DE), C_7/3-phth (IDT, Melbourne, Australia), PZP (Thomae, Biberach an der Riss, Germany), McN-A-343 (Research Biochemicals Inc., Natick, MA), and CCh, NMS, and atropine sulfate (Sigma Chemical Co., St. Louis, MO).

Data evaluation. Data are given as means ± standard errors. Statistical significance was determined using Student's *t* test, with values of $p < 0.05$ being considered significant.

Data from radioligand kinetic studies were fitted via nonlinear regression analysis using the computer program Fig.P 6.0 or Fig.P for Windows 1.2 (Biosoft). The slope of each line analyzed in this manner represented the dissociation rate constant (k_{off}) of the radioligand under the different conditions studied.

In organ bath experiments, DRs were calculated from the shifts of the curves. The shift occurring in the presence of C_7/3-phth and either NMS, PZP, or atropine was compared with that expected from such a combination if the antagonists were acting competitively, based on the experimental shift obtained with the competitive antagonist plus the shift that a concentration of 10 μM C_7/3-phth alone produced in separate experiments. The cooperativity factor (α') for interaction between C_7/3-phth and a competitive antagonist was calculated from these experiments using a plot of log (DR_{XB} − 1) versus log (DR_{XAB} − DR_{XA}), where DR_{XB}, DR_{XA}, and DR_{XAB} are the DRs produced by the competitive antagonist, the allosteric antagonist, and the combination of the two antagonists, respectively. The intercept of this plot with the *x*-axis was used to calculate α' from the following relationship, which is derived in the Appendix:

$$\alpha' = \frac{[A] \times DR_{XA} \times I}{[A] + K_A(1 - (DR_{XA} \times I))}$$

where I is the antilogarithm of the $-x$-intercept, K_A is the dissociation constant of the allosteric antagonist, and [A] is the concentration of the allosteric antagonist.

Results

Radioligand kinetic studies in atria. The dissociation rate of [^{3}H]NMS (0.3 nM) was studied in guinea pig atrial homogenates in the absence and presence of C_7/3-phth (10 μM). The k_{off} of [^{3}H]NMS, measured in the presence of 9 nM unlabeled NMS (~30 times the K_d) alone was 0.95 ± 0.09 min^{-1} (four experiments). In the presence of C_7/3-phth (10 μM, ~140 times the K_i) alone, no dissociation of [^{3}H]NMS was evident over the course of 10 min (Fig. 1). Combination of 9 nM unlabeled NMS with 10 μM C_7/3-phth also resulted in no evident effect on the dissociation rate of [^{3}H]NMS (Fig. 1).

Radioligand kinetic studies in cortex. Dissociation of [^{3}H]PZP (3 nM) was studied in rat cerebral cortex using unlabeled 90 nM PZP (~30 times the K_d) and C_7/3-phth (10 μM), alone or in combination. In each instance, the dissociation rate of [^{3}H]PZP was affected to the same extent by unlabeled PZP, C_7/3-phth, or the combination of the two (Fig. 2). The k_{off} values were 0.39 ± 0.01, 0.40 ± 0.01, and 0.38 ± 0.01 (three experiments), respectively.

The rate of dissociation of [^{3}H]NMS was similarly studied in rat cerebral cortex using unlabeled NMS at either 9 nM or 30 nM and C_7/3-phth at 10 μM, alone or in combination. In each instance, the dissociation of [^{3}H]NMS was biphasic (Fig. 3); the k_{off} for each phase is shown in Table 1.

The dissociation rate of [^{3}H]QNB in rat cortex was also studied in the presence of either 1.35 nM or 4.5 nM unlabeled QNB and appeared to be monophasic. The addition of C_7/3-phth (10 μM) resulted in a slowing of the dissociation rate of the radioligand (Fig. 4; Table 2).

Organ bath antagonist combination experiments in guinea pig atria. C_7/3-phth (10 μM) alone produced a rightward shift of the CCh C/R curve with a mean DR of 88.09 (range, 32.01–242; five experiments), corresponding to an apparent K_B value of 115.2 nM (range, 41.4–320.6 nM). The inhibition by NMS of responses to CCh was enhanced in the presence of C_7/3-phth (10 μM). As can be seen in Fig. 5A, the 615.4-fold shift of the CCh C/R curve induced by NMS (30

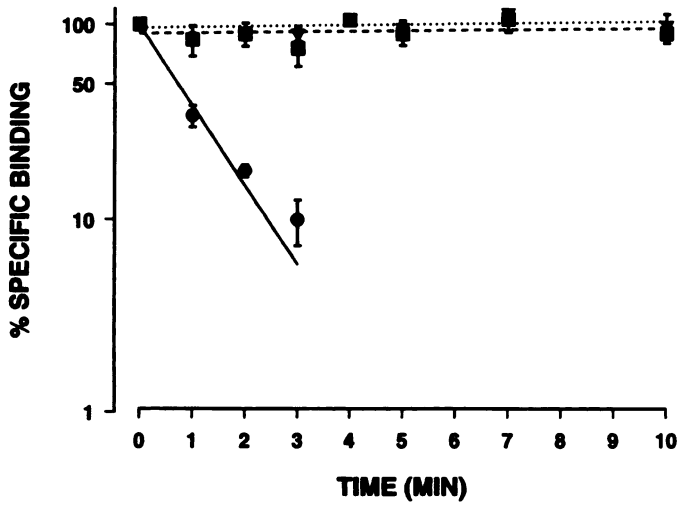

Fig. 1. Effect of C_7/3-phth on the dissociation rate of [^{3}H]NMS in guinea pig atria. Homogenates were preincubated with 0.3 nM [^{3}H]NMS in 50 mM phosphate buffer, pH 7.4, at 32° for 1 hr before dissociation was followed from time 0 via the addition of NMS (9 nM) (●), NMS (9 nM) plus C_7/3-phth (10 μM) (■), or C_7/3-phth (10 μM) (▼). Each *point* represents the mean of four to eight experiments conducted in duplicate.

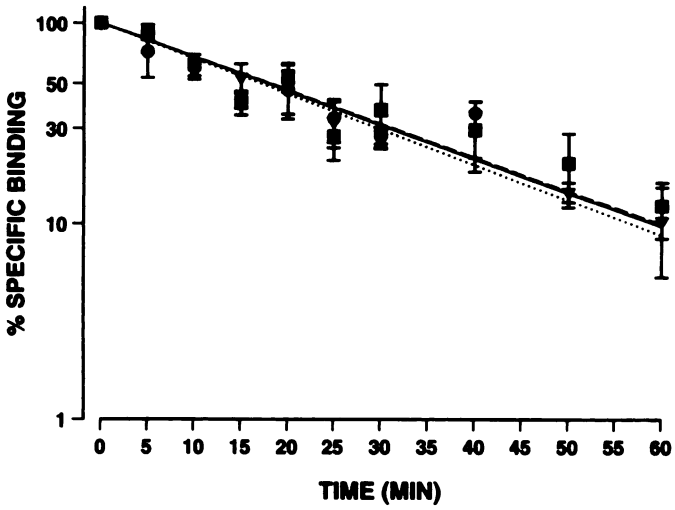

Fig. 2. Effect of C_7/3-phth on the dissociation rate of [^{3}H]PZP in rat cerebral cortex. Homogenates were preincubated with 3 nM [^{3}H]PZP, as indicated in Fig. 1, before dissociation was followed from time 0 via the addition of PZP (90 nM) (●), PZP (90 nM) plus C_7/3-phth (10 μM) (■), or C_7/3-phth (10 μM) (▼). Each *point* represents the mean of three experiments conducted in duplicate.

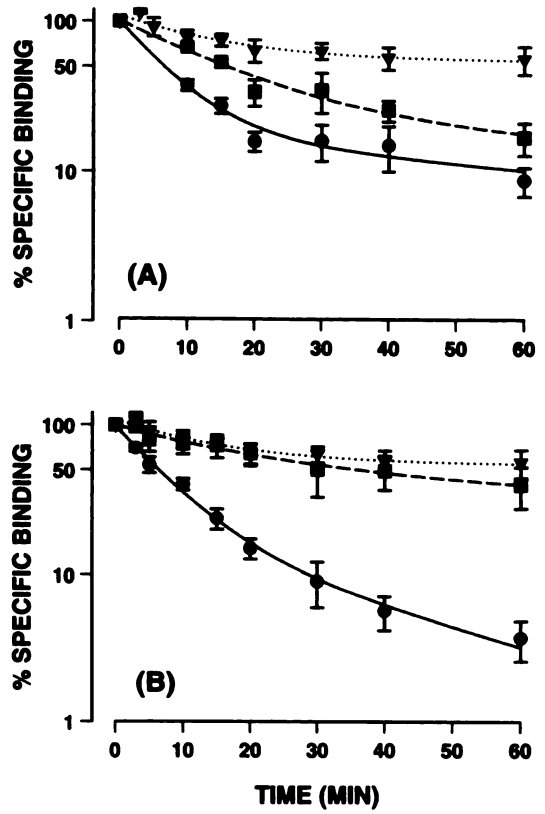

Fig. 3. Effect of C_7/3-phth on the dissociation rate of [^{3}H]NMS in rat cerebral cortex. Homogenates were preincubated as indicated in Fig. 1 before dissociation was followed from time 0 via the addition of NMS (9 nM) (●), NMS (9 nM) plus C_7/3-phth (10 μM) (■), or C_7/3-phth (10 μM) (▼) (A) or NMS (30 nM) (●), NMS (30 nM) plus C_7/3-phth (10 μM) (■), or C_7/3-phth (10 μM) (▼) (B). Each *point* represents the mean of four to eight experiments performed in duplicate.

TABLE 1

Dissociation rate parameters for [^{3}H]NMS (0.3 nM) in the presence of NMS and C_7/3-phth, alone or in combination, in rat cerebral cortex

[NMS]	[C_7/3-phth]	n^a	k_{off} fast^b	k_{off} slow^c
nM	μM		min^{-1}	min^{-1}
	10	8	0.051 ± 0.009	0 ± 0.017^d
9		4	0.114 ± 0.034	0.014 ± 0.005
9	10	4	0.086 ± 0.013	0.013 ± 0.005
30		4	0.133 ± 0.008	0.031 ± 0.006
30	10	4	0.054 ± 0.009	0.005 ± 0.002

[a] Number of experiments.
[b] Dissociation rate of [^{3}H]NMS (fast dissociation component), as determined by the program Fig. P (see Data evaluation).
[c] Dissociation rate of [^{3}H]NMS (slow dissociation component), as determined by the program Fig. P.
[d] Computer analysis of the data gave a value of −0.006 ± 0.002. The value shown is that obtained when the dissociation rate constant is constrained to values greater than or equal to 0.

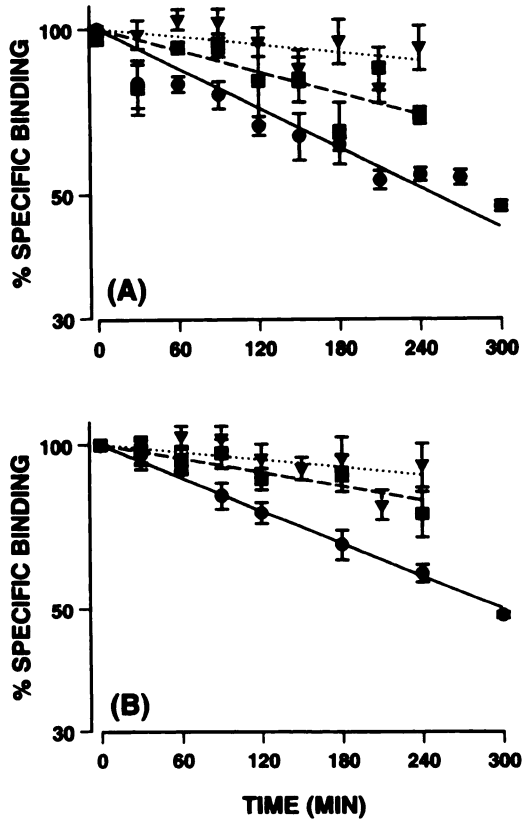

Fig. 4. Effect of C_7/3-phth on the dissociation rate of [^{3}H]QNB in rat cerebral cortex. Homogenates were preincubated with 50 pM [^{3}H]QNB, as indicated in Fig. 1, before dissociation was followed from time 0 via the addition of QNB (1.35 nM) (●), QNB (1.35 nM) plus C_7/3-phth (10 μM) (■), or C_7/3-phth (10 μM) (▼) (A) or QNB (4.5 nM) (●), QNB (4.5 nM) plus C_7/3-phth (10 μM) (■), or C_7/3-phth (10 μM) (▼) (B). Each *point* represents the mean of three to eight experiments performed in duplicate.

nM) alone was potentiated by C_7/3-phth. The expected shift for a combination of two competitive antagonists (Fig. 5A, *dashed line*) was 702.4-fold (615.4 + 88.09 − 1); however, the combination caused an observed shift of ~5120-fold.

To ascertain whether C_7/3-phth would interact allosterically with PZP in this tissue, similar functional studies were used. In this case, the degree of supra-additivity was not as pronounced (Fig. 5B). Experiments with a range of concentrations of NMS (3–30 nM) or PZP (5–30 μM) in combination with C_7/3-phth (10 μM) showed that the degree of supra-additivity was greater for NMS (10.04-fold) than for PZP (2.61-fold) (Fig. 6).

Organ bath washout experiments in guinea pig atria. Studies were undertaken to measure the rate of removal of NMS from the isolated atria after equilibrium had been established and to observe whether this rate of removal was inhibited by C_7/3-phth. The degree of receptor occupancy was determined by establishing the concentration of CCh required to produce an "apparent" EC_{50} response at various times after washout (see Materials and Methods). C_7/3-phth (10 μM) slowed the washout of NMS by a factor of 1.76 ± 0.43 (three experiments) (Fig. 7). The time taken for receptor occupancy to decline from 99% (DR = 100) to 90% (DR = 10) was 16.7 ± 1.7 min (three experiments) for NMS alone and 46.7 ± 1.7 min (three experiments) for the combination.

Organ bath antagonist combination experiments in rabbit vas deferens. The effect of C_7/3-phth on PZP and atropine at the M_1 receptor was investigated in a series of combination experiments in rabbit vas deferens. In these experiments, addition of C_7/3-phth (10 μM) caused the effect of atropine on the McN-A-343 C/R curve to be increased 2.29 ± 0.26-fold (five experiments) over that expected for a combination of two competitive antagonists (Fig. 8A). When PZP was used instead of atropine, addition of C_7/3-phth (10 μM) failed to cause any additional rightward shift of the McN-A-343 C/R curve; rather, a small leftward shift was observed (supra-additivity factor, 0.53 ± 0.14; six experiments) (Fig. 8B).

Discussion

The experiments reported here reveal marked differences in the ability of C_7/3-phth to interact allosterically with muscarine receptor ligands, depending on the receptor subtype involved as well as the ligand used. In atria, [^{3}H]NMS dissociation, after addition of unlabeled NMS (~30 times the K_d), was almost complete (<10% specific binding) within 5 min and appeared to be monophasic, in agreement with reports by others (16, 17). In contrast, no dissociation of [^{3}H]NMS was observed in the presence of 10 μM C_7/3-phth alone or in combination with unlabeled NMS, indicating an allosteric mode of action for C_7/3-phth at the M_2 receptor. The concentration of C_7/3-phth used in these experiments corresponded to ~140 times the K_i at the M_2 muscarine receptor (5), so the failure of [^{3}H]NMS to dissociate in the presence of C_7/3-phth alone further supports an allosteric interaction. The analogous hexamethylene derivative of C_7/3-phth, W84, has also been shown to produce a marked slowing of [^{3}H]NMS dissociation in cardiac tissue (16).

In functional experiments, a combination of two competitive antagonists should produce a DR equivalent to the sum of the

TABLE 2

Dissociation parameters for [^{3}H]QNB (50 pM) in the presence of QNB and C_7/3-phth, alone or in combination, in rat cerebral cortex

[QNB]	[C_7/3-phth]	n^a	$k_{off} \times 10^{-4b}$
nM	μM		min^{-1}
	10	8	5.8 ± 1.7
1.35		3–7	27.8 ± 1.7
1.35	10	4	15.2 ± 2.7
4.5		3–7	23.1 ± 0.6
4.5	10	4	9.6 ± 1.2

[a] Number of experiments.
[b] Dissociation rate of [^{3}H]QNB, as determined by the program Fig. P.

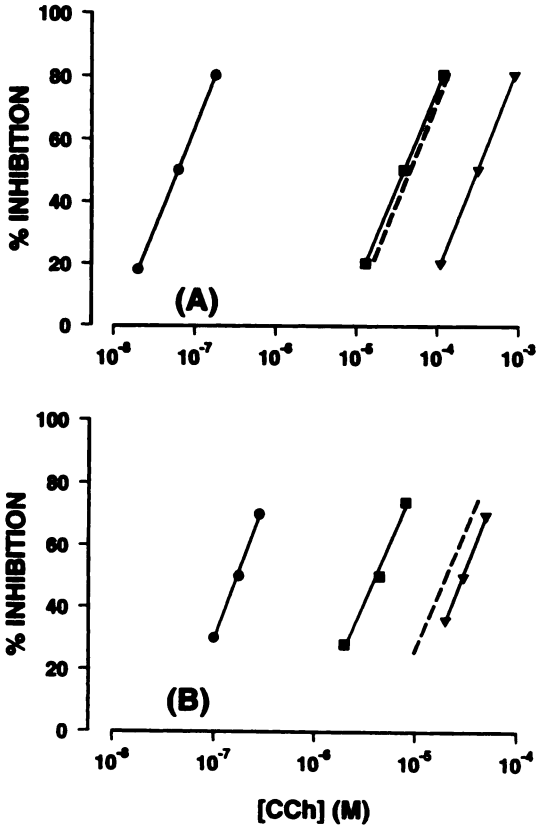

Fig. 5. Representative experiments on the effect of NMS and PZP on the C/R curve for CCh (●) in guinea pig atria. A, Shift of the CCh C/R curve produced by NMS (30 nM) in the absence (■) and presence (▼) of C_7/3-phth (10 μM). B, Shift of the CCh C/R curve produced by PZP (30 μM) in the absence (■) and presence (▼) of C_7/3-phth (10 μM). *Dashed line*, shift expected for the combination of the two antagonists if the nature of their interaction was competitive. Experiments were conducted at 37° using McEwen's solution.

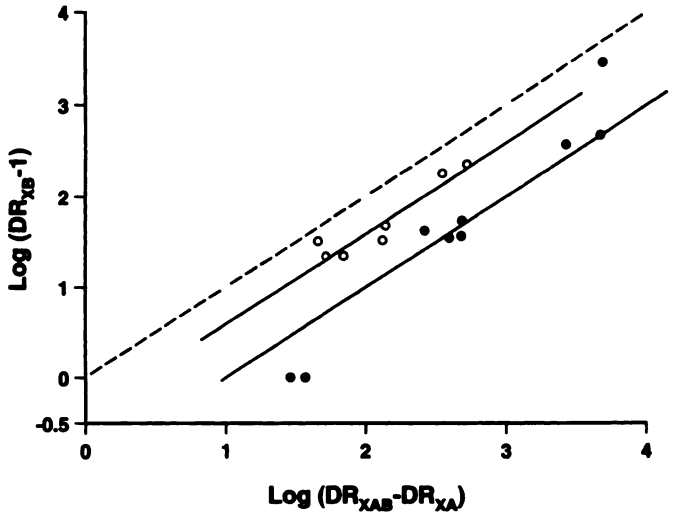

Fig. 6. Relationship between DR − 1 for a competitive antagonist alone (DR_{XB} − 1) and DR for the combination of a competitive antagonist and an allosteric antagonist − DR for the allosteric antagonist (DR_{XAB} − DR_{XA}). Both coordinates are expressed on a logarithmic scale. Each *point* is the result of a single experiment using a single concentration of either NMS (●; within the range of 3–30 nM) or PZP (○; within the range of 5–30 μM) as the competitive antagonist, in conjunction with C_7/3-phth (10 μM). For two competitive antagonists the data should lie along the *dashed line*, but for an allosteric antagonist in combination with a competitive antagonist the relationship is displaced to the right in a parallel fashion.

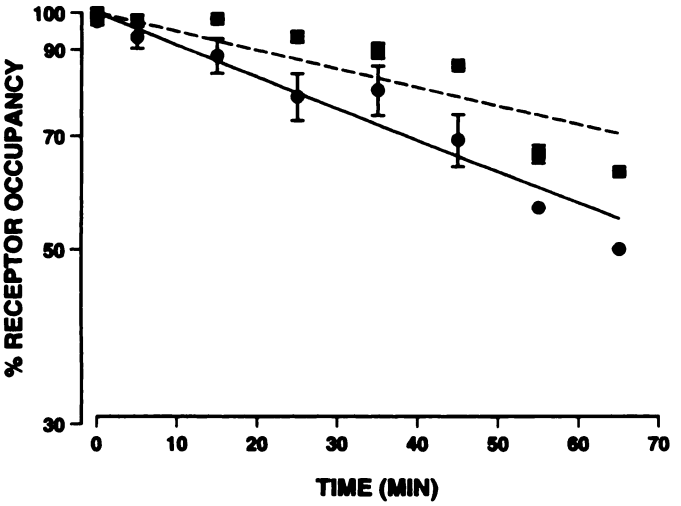

Fig. 7. Time course of washout of NMS alone (●) or in combination with C_7/3-phth (10 μM) (■) from electrically driven guinea pig atria, as assessed by the negative inotropic effect of CCh. The decreases in the "apparent" EC_{50} values of CCh over time were calculated as DRs (compared with the control EC_{50}), to assess the percentage receptor occupancy of NMS. Each *point* represents the mean of three experiments. Experiments were conducted at 37° using McEwen's solution.

DRs produced by each antagonist alone − 1 (18). The studies in atria using the combination of NMS and C_7/3-phth revealed a degree of inhibition of responses to CCh far greater than expected for the combination of two competitive antagonists. This supra-additivity could be explained if C_7/3-phth was acting to slow the dissociation rate of NMS to a greater extent than that of the agonist CCh. In this respect, it has been demonstrated that the congener W84 failed to inhibit the rate of [^{3}H]oxotremorine-M dissociation from cardiac membranes, in contrast to its marked effects on [^{3}H]NMS dissociation (19).

Washout experiments in isolated atria also suggested that the dissociation of NMS was slowed in the presence of C_7/3-phth. Lüllmann *et al.* (20) have shown that the $t_{1/2}$ values for washout do not correspond to those for dissociation because during the former procedure the drug can rebind while diffusing through the biophase. However, differences between the times in the absence and presence of C_7/3-phth reflect differences in the dissociation of NMS. The times for the DR to decline from 100 to 10 (receptor occupancies of 99% and 90%, respectively)

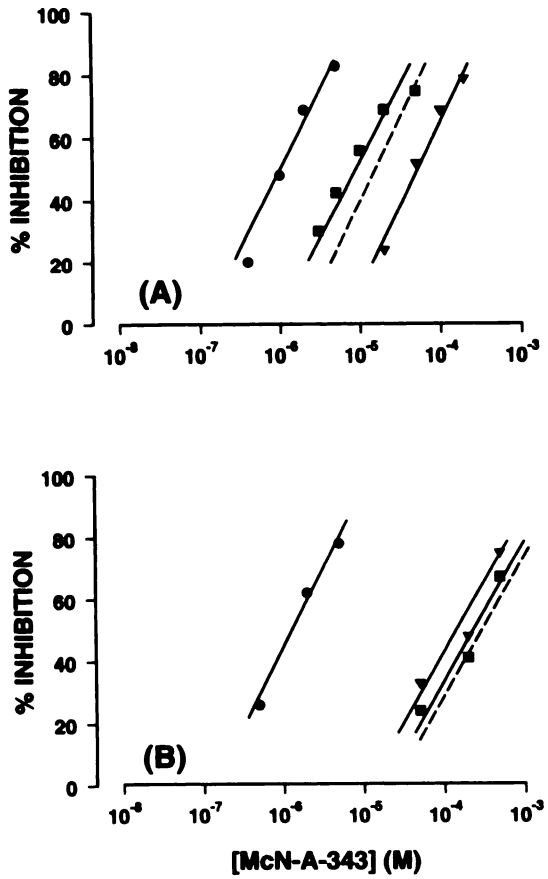

Fig. 8. Representative experiments on the effect of atropine and PZP on the C/R curve for McN-A-343 (●) in rabbit vas deferens. A, Shift of the McN-A-343 C/R curve produced by atropine (10 nM) in the absence (■) and presence (▼) of C_7/3-phth (10 μM). B, Shift of the McN-A-343 C/R curve produced by PZP (300 nM) in the absence (■) and presence (▼) of C_7/3-phth (10 μM). *Dashed line*, shift expected for the combination of the two antagonists if the nature of their interaction was competitive. Experiments were conducted at 37° using Eltze's solution (15).

were 2.8-fold slower and significantly different ($p < 0.01$) in the preparations where C_7/3-phth was present.

Ehlert (21) has defined the term α (cooperativity factor) as the magnitude by which two drugs that act allosterically at heterotropic sites alter their respective dissociation constants. For the situation where the allosteric ligand affects only the affinity and not the efficacy of an agonist, it is possible to write (rearranging eq. 23 in Ref. 21)

$$DR = \frac{K_A + [A]}{K_A + \frac{[A]}{\alpha}}$$

where K_A is the dissociation constant and [A] is the concentration of the allosteric antagonist. The term α in this equation is equivalent to the term $1 - \kappa$ derived for an allosteric antagonist in an earlier theoretical treatment of allosteric interaction at receptor sites by Ariëns *et al.* (22), because the latter group derived the expression

$$DR = \frac{K_A + [A]}{K_A + \frac{[A]}{(1 - \kappa)}}$$

The value of κ for C_7/3-phth interacting with CCh has been estimated as −6470 (2), leading to the value of α being 6471, indicating that C_7/3-phth and CCh exhibited a high degree of negative heterotropic cooperativity.

The 10-fold degree of supra-additivity observed with NMS at cardiac M_2 muscarine receptors was 5 times greater than that observed with PZP in the same tissue (Fig. 6), indicating that C_7/3-phth allosterically modifies the dissociation of NMS differently from that of PZP. A likely explanation is that PZP and C_7/3-phth interact with a high degree of negative heterotropic cooperativity, greater than that between NMS and C_7/3-phth. Other allosteric antagonists have also been shown to differentiate between NMS and PZP at other muscarine receptor sites. For example, Gillard *et al.* (23) showed that gallamine induced greater slowing of the dissociation of [^{3}H]NMS at cortical muscarine receptors, compared with that of [^{3}H]PZP.

Consideration of the possible heterotropic interaction between an allosteric antagonist and a competitive antagonist, as well as an agonist (where only the affinity of the agonist was affected by the allosteric antagonist), showed that α', the cooperativity factor between the allosteric antagonist and the competitive antagonist, could be derived from the combination DR experiments (see Appendix). Evaluation of the effect of NMS by this technique provided a value of 8.8 ± 1.6 (eight experiments) for α'. For PZP, the value was 58.2 ± 12.8 (seven experiments) (Table 3). The value of α' is also equivalent to $1 - \kappa'$ in the theoretical derivation of Ariëns *et al.* (22), so that in Table 3 it can be seen that PZP displayed the highest degree of negative heterotropic cooperativity with C_7/3-phth, compared with NMS and the other competitive antagonists for which κ' has been estimated (2), and the values of the cooperativity factors for all of the antagonists are much lower than that for the agonist CCh.

Studies conducted with C_7/3-phth at the M_1 muscarine receptor in the rat cortex also showed that PZP was affected differently from the other muscarine receptor antagonists. The dissociation of [^{3}H]PZP from the binding site in the presence of unlabeled PZP (~30 times the K_d) was not significantly different ($p < 0.01$) from that in the presence of C_7/3-phth alone (~30 times the K_i) or when combined with C_7/3-phth. This behavior is suggestive of a competitive rather than an allosteric interaction but may be due to C_7/3-phth and PZP interacting allosterically with a high degree of negative heterotropic cooperativity, as suggested above for the M_2 receptor. Further experiments are required to distinguish between the two possibilities. In contrast, slowing of the dissociation of [^{3}H]QNB or [^{3}H]NMS was evident in the presence of C_7/3-phth alone or in combination with unlabeled QNB or unlabeled NMS, respectively, clearly indicating an allosteric mechanism.

Ellis *et al.* (24) have recently suggested that α may be evaluated by estimating the concentration of allosteric ligand causing a half-maximal slowing of the dissociation rate of a competitive antagonist in the presence of an excess of unlabeled ligand. With the one concentration of C_7/3-phth used (10 μM) this was not possible in atria, because virtually complete inhibition of the dissociation of [^{3}H]NMS was achieved. However, assuming that complete inhibition of radioligand dissociation may be obtained with higher concentrations of C_7/3-phth, it was possible to approximate a value of α by this method at the M_1 receptor in the cerebral cortex. The combination of C_7/3-phth (10 μM) with [^{3}H]QNB or [^{3}H]NMS reduced the dissociation rate to approximately half that in the control. Calculation

TABLE 3

Estimates of cooperativity factors for C_7/3-phth at the cardiac M_2 receptor with a number of muscarine receptor ligands

Factors for the antagonists were determined in the presence of CCh.

Ligand	α[a]	α'[a]
CCh	6471[b]	
Atropine		33.1 ± 6.3[b]
Homatropine		39.7 ± 5.0[b]
Dexetimide		25.9 ± 4.0[b]
NMS		8.8 ± 1.6
PZP		58.2 ± 12.8

[a] Cooperativity factor, based on calculations outlined in Data evaluation.
[b] Recalculated from Ref. 2.

of α (α' in our case) from the relationship (24)

$$K_{app} = \alpha K_B$$

gave an α value of 31.8 when a value of 10 μM was used for the K_{app} of C_7/3-phth. On this point, it is worth noting that the change in dissociation rate of a ^{3}H-labeled ligand in the presence of an excess of competitive antagonist is only useful for detecting allosteric compounds with limited heterotropic cooperativity; highly cooperative interactions are difficult to distinguish from competitive inhibition or from noninteracting drugs.

It is often suggested that allosteric antagonists such as gallamine interact competitively at low concentrations and allosterically at high concentrations (25–28). This did not appear to be the case with C_7/3-phth at M_2 receptors in atria, because this compound, at a concentration of 10 μM (equivalent to ~140 times the K_i), failed to reveal any dissociation of [^{3}H]NMS. If it were acting competitively, it should have allowed [^{3}H]NMS to show a dissociation rate similar to that observed in the presence of 9 nM unlabeled NMS alone (~100 times the K_d).

In the kinetic experiments with [^{3}H]QNB in the cerebral cortex, the ^{3}H-ligand dissociated slowly and in a monophasic fashion. The majority of receptors recognized by this compound are of the M_1 subtype (5), and the addition of C_7/3-phth resulted in a ~2.4-fold slowing of the dissociation rate of the radioligand in the presence of unlabeled QNB (100 times the K_d). With [^{3}H]NMS, the dissociation of the radioligand proceeded in a biphasic manner, as previously reported by Waelbroeck *et al.* (17), who suggested that the initial rapid phase of dissociation represented offset from M_1 (major proportion) and M_2 receptors, whereas the later slow phase of dissociation represented offset from M_3 and M_4 (major proportion) receptors. Other reports have also shown that the quaternary NMS appears to recognize greater receptor heterogeneity than does the tertiary QNB (29, 30). A comparison of the slowing in dissociation produced by C_7/3-phth in combination with 9 nM (~100 times the K_d) unlabeled NMS during the first phase of dissociation with that produced by the compound in combination with 4.5 nM (~100 times the K_d) unlabeled QNB revealed similar results (Tables 1 and 2). If the M_2 receptors recognized by [^{3}H]NMS in the rapid dissociation phase constituted a significant proportion, a much greater degree of slowing would have been expected, as evidenced by the findings in cardiac membranes. This was not the case, and thus it was considered that the dissociation in this phase involved the M_1 receptor.

The second phase of the [^{3}H]NMS dissociation in the presence of 30 nM (~300 times the K_d) unlabeled NMS was slowed 6.2-fold with the addition of C_7/3-phth, suggesting that the allosteric effect of C_7/3-phth at the M_4 receptor was more obvious than that at the M_1 receptor, although still not as evident as that observed at the cardiac M_2 receptor.

Functional experiments similar to those with the M_2 receptor were conducted with peripheral M_1 receptors of the rabbit vas deferens (15). As was the case with NMS in atria, the combination of C_7/3-phth and the nonselective antagonist atropine resulted in a greater degree of inhibition of the responses to the agonist McN-A-343 than expected for two competitive antagonists, with the α' value for atropine being estimated as 7.2 ± 1.9 (five experiments). When PZP was used in place of atropine, no supra-additivity was evident. The small degree of leftward shift in the C/R curve for McN-A-343 after addition of C_7/3-phth in the presence of PZP may be due to the high concentration of agonist (in the presence of PZP) having some action on the postjunctional M_2 facilitatory receptor. Consequently, in the presence of C_7/3-phth, which has a 3-fold selectivity for the M_2 receptor in this preparation (4), the resultant effect was a small leftward shift rather than a small rightward one.

As mentioned previously, kinetic studies with gallamine have shown that it produces a more pronounced slowing of the dissociation of [^{3}H]NMS than of [^{3}H]PZP (23) and has a greater apparent effect on quaternary, compared with tertiary, antagonists (25, 27) and on tropates, compared with benzilates (27). It is of interest that the calculated values of the cooperativity factors in binding experiments with gallamine at M_2 receptors show a similar trend as do those estimated with C_7/3-phth in functional studies. Interaction of gallamine with competitive antagonists such as NMS and atropine gave low negative cooperativity factors, 14 and 28, respectively, whereas with PZP the factor was 88, a value comparable to the value of 78 for CCh (31). In functional studies values of 21 for NMS (32) and 192 for CCh (33) were obtained. In the cerebral cortex, the values of the cooperativity factors were ~50% lower for NMS and atropine but were almost unaltered for PZP or CCh (31). The striking difference between C_7/3-phth and gallamine is the high degree of negative heterotropic cooperativity exerted by C_7/3-phth with agonists such as CCh, and this fact, coupled with the low degree of negative heterotropic cooperativity with antagonists such as NMS or atropine, may lead to marked supra-additivity, as observed in this study.

In conclusion, this study has demonstrated that the allosteric effects of C_7/3-phth vary with the receptor subtype (M_1 or M_2), and this can be manifested in functional equilibrium experiments or radioligand kinetic experiments. Furthermore, it is evident that the allosteric inhibitor modulates binding of NMS, QNB, and atropine differently from that of PZP at either M_1 or M_2 sites. This was shown in either kinetic or functional studies at the two receptor subtypes. Although it was possible to study both dissociation kinetics and supra-additivity in functional experiments on the M_2 receptor with NMS, only the latter type of experiment was possible with PZP because of its low affinity for the M_2 receptor. Nevertheless, the qualitative correlation for NMS between kinetic and functional studies suggests that the latter studies with PZP support this conclusion.

Appendix

A scheme describing the interaction between an agonist (X), a competitive antagonist (B), and an allosteric antagonist (A) may be given as

$$\begin{array}{ccccc} [RB] + [A] + [X] & \overset{K_B}{\rightleftharpoons} & [R] + [X] + [B] + [A] & \overset{K_X}{\rightleftharpoons} & [XR] + [B] + [A] \\ \Updownarrow \alpha' K_A & & \Updownarrow K_A & & \Updownarrow \alpha K_A \\ [ARB] + [X] & \overset{\alpha' K_B}{\rightleftharpoons} & [RA] + [X] + [B] & \overset{\alpha K_X}{\rightleftharpoons} & [XRA] + [B] \end{array}$$

where [RB], [RA], and [XR] denote the drug-receptor complexes formed, [ARB] and [XRA] denote the ternary complexes formed, and K_X, K_B, and K_A denote the dissociation constants of the agonist, competitive antagonist, and allosteric antagonist for their respective sites on the receptor complex. The magnitude of the heterotropic interaction between the allosteric antagonist and the agonist is denoted by α and that between the allosteric antagonist and the competitive antagonist is denoted by α'. The dissociation constants may be defined as follows:

$$K_X = \frac{[R][X]}{[XR]} \quad \text{(A1)}$$

$$K_B = \frac{[R][B]}{[RB]} \quad \text{(A2)}$$

$$K_A = \frac{[R][A]}{[RA]} \quad \text{(A3)}$$

$$\alpha K_X = \frac{[RA][X]}{[XRA]} \quad \text{(A4)}$$

$$\alpha K_A = \frac{[XR][A]}{[ARX]} \quad \text{(A5)}$$

$$\alpha' K_B = \frac{[RA][B]}{[ARB]} \quad \text{(A6)}$$

$$\alpha' K_A = \frac{[RB][A]}{[ARB]} \quad \text{(A7)}$$

and the total receptors ($[R_T]$) may be given as

$$[R_T] = [R] + [XR] + [RA] + [RB] + [XRA] + [ARB] \quad \text{(A8)}$$

The binding of the agonist X in the presence of both A and B may be expressed as

$$\frac{[XR] + [XRA]}{[R_T]} = \frac{[XR] + [XRA]}{[R] + [XR] + [RA] + [RB] + [XRA] + [ARB]} \quad \text{(A9)}$$

Multiplication of the numerator and denominator of the fraction on the right side of the equation by $1/[X][R]$ yields

$$\frac{[XR] + [XRA]}{[R_T]} = \frac{\dfrac{[XR]}{[X][R]} + \dfrac{[XRA]}{[X][R]}}{\dfrac{[R]}{[X][R]} + \dfrac{[XR]}{[X][R]} + \dfrac{[RA]}{[X][R]} + \dfrac{[RB]}{[X][R]} + \dfrac{[XRA]}{[X][R]} + \dfrac{[ARB]}{[X][R]}} \quad \text{(A10)}$$

The following substitutions may then be made where appropriate:

$$\frac{[XR]}{[X][R]} = \frac{1}{K_X}$$

$$[XRA] = \frac{[XR][A]}{\alpha K_A}$$

$$[R] = \frac{K_A[RA]}{[A]} = \frac{K_B[RB]}{[B]}$$

$$[ARB] = \frac{[RB][A]}{\alpha' K_A}$$

and the equation can be subsequently expressed as

$$\frac{[XR] + [XRA]}{[R_T]} = \frac{\dfrac{1}{K_X} + \dfrac{[A]}{\alpha K_A K_X}}{\dfrac{1}{[X]} + \dfrac{1}{K_X} + \dfrac{[B]}{[X]K_B} + \dfrac{[A]}{[X]K_A} + \dfrac{[A]}{\alpha K_A K_X} + \dfrac{[A][B]}{\alpha'[X]K_A K_B}} \quad \text{(A11)}$$

This equation simplifies to

$$\frac{[XR] + [XRA]}{[R_T]} = \frac{[X]}{[X] + K_X''} \quad \text{(A12)}$$

or

$$[XR] + [XRA] = \frac{[X][R_T]}{[X] + K_X''} \quad \text{(A13)}$$

where

$$K_X'' = \frac{\alpha K_A K_X}{\alpha K_A + [A]}\left(1 + \frac{[B]}{K_B} + \frac{[A]}{K_A} + \frac{[A][B]}{\alpha' K_A K_B}\right)$$

If it is assumed that the allosteric antagonist A does not affect the intrinsic efficacy of the agonist X, then the relationship between concentrations of the agonist producing equipotent responses in the absence (X) and presence (X″) of the combination of antagonists may be given as

$$\frac{[X][R_T]}{[X] + K_X} = \frac{[X''][R_T]}{[X''] + K_X''} \quad \text{(A14)}$$

Solving for X″,

$$[X''] = \frac{K_X''[X]}{K_X} \quad \text{(A15)}$$

or, alternatively,

$$\frac{[X'']}{[X]} = \frac{K_X''}{K_X} \quad \text{(A16)}$$

When the response measured is the EC_{50} response, then

$$\frac{[X'']}{[X]} = \frac{[EC_{50}'']}{[EC_{50}]} = DR_{XAB} = \frac{K_X''}{K_X} \quad \text{(A17)}$$

that is, DR_{XAB} represents the DR for the agonist X observed in the presence of the combination of both A and B. Substituting for K_X'' thus yields

$$DR_{XAB} = \frac{\alpha K_A}{\alpha K_A + [A]}\left(1 + \frac{[B]}{K_B} + \frac{[A]}{K_A} + \frac{[A][B]}{\alpha' K_A K_B}\right) \quad \text{(A18)}$$

Paton and Rang (18) showed that for a combination of two competitive antagonists, B and C, against agonist X the combination DR was

$$DR_{XBC} = DR_{XB} + DR_{XC} - 1 \quad \text{(A19)}$$

where DR_{XB} and DR_{XC} are the DRs produced by each respective antagonist alone and

$$\log(DR_{XB} - 1) = \log(DR_{XBC} - DR_{XC}) \quad \text{(A20)}$$

Thus, in this case, a plot of log $(DR_{XB} - 1)$ versus log $(DR_{XBC} - DR_{XC})$ yields a linear relationship passing through the origin. A similar relationship for combination of an allosteric antagonist and competitive antagonist may be derived from eq. A18. Ehlert (21) has previously defined the DR for the agonist X in the presence of the allosteric antagonist A, and this may be expressed as

$$DR_{XA} = \frac{\alpha K_A}{\alpha K_A + [A]}\left(1 + \frac{[A]}{K_A}\right) \quad \text{(A21)}$$

Also, the DR of X in the presence of the competitive antagonist B has been defined (34) as

$$DR_{XB} = \frac{[B]}{K_B} + 1 \quad \text{(A22)}$$

Hence, by making the appropriate substitutions into eq. A18, it can be shown that

$$DR_{XB} - 1 = \left(\frac{\frac{[A]}{\alpha K_A} + 1}{\frac{[A]}{\alpha' K_A} + 1}\right)(DR_{XAB} - DR_{XA}) \quad \text{(A23)}$$

Alternatively,

$$\log(DR_{XB} - 1) = \log(DR_{XAB} - DR_{XA}) + \log\left(\frac{\frac{[A]}{\alpha K_A} + 1}{\frac{[A]}{\alpha' K_A} + 1}\right) \quad \text{(A24)}$$

and

$$\log(DR_{XB} - 1) = \log(DR_{XAB} - DR_{XA}) + \log\frac{\frac{[A]}{K_A} + 1}{DR_{XA}\left(\frac{[A]}{\alpha' K_A} + 1\right)} \quad \text{(A25)}$$

Thus, a plot of log $(DR_{XB} - 1)$ versus log $(DR_{XAB} - DR_{XA})$ yields a linear relationship with unit slope, with the x-axis intercept being

$$-\log \frac{\frac{[A]}{K_A} + 1}{DR_{XA}\left(\frac{[A]}{\alpha' K_A} + 1\right)}$$

from which α' may be estimated according to the following relationship:

$$\alpha' = \frac{[A] \times DR_{XA} \times I}{[A] + K_A(1 - (DR_{XA} \times I))} \quad \text{(A26)}$$

where I is the the antilogarithm of the $-x$-intercept.

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